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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/00, 15/01, A01H 1/00, 1/06, 5/00</b>		<b>A1</b>	(11) International Publication Number: <b>WO 99/02668</b>
			(43) International Publication Date: <b>21 January 1999 (21.01.99)</b>
(21) International Application Number: <b>PCT/US98/13685</b>		(71)(72) Applicants and Inventors: <b>CHANG, Ming-Tang</b> [US/US]; Exseed Genetics LLC, Iowa State University, 1573 Food Science Building, Ames, IA 50011-1061 (US). <b>KEELING, Peter, L.</b> [GB/US]; Exseed Genetics LLC, Iowa State University, 1573 Food Science Building, Ames, IA 50011-1061 (US). <b>WILHELM, Edward, P.</b> [US/US]; Exseed Genetics LLC, Iowa State University, 1573 Food Science Building, Ames, IA 50011-1061 (US). <b>WEIGEL, Jerry, C.</b> [US/US]; Exseed Genetics LLC, Suite 345C, 363 So. Main Street, Decatur, IL 62523 (US).	
(22) International Filing Date: <b>7 July 1998 (07.07.98)</b>		(74) Agent: <b>SADOFF, B., J.; Nixon &amp; Vanderhye, P.C.,</b> 8th floor, 1100 North Glebe Road, Arlington, VA 22201 (US).	
(30) Priority Data: 60/051,854 7 July 1997 (07.07.97) US 60/051,855 7 July 1997 (07.07.97) US		(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>	
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications US 60/051,854 (CIP) Filed on 7 July 1997 (07.07.97) US 60/051,855 (CIP) Filed on 7 July 1997 (07.07.97)		<b>Published</b> <i>With international search report.</i> <i>With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.</i>	
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(54) Title: <b>ANIMAL FEED WITH LOW PHYTIC ACID, OIL BURDENED AND PROTEIN LADEN GRAIN</b>			
(57) Abstract <p>The present invention provides grain, seed, feed made from the grain or seed, petfood made from the grain, and food products made from the grain. The grain may be maize grain with the following characteristics: oil burdened, elevated protein content, and low phytate levels. The combination of oil burdened, protein laden, decreased phytate characteristics in grain makes a grain that provides more calories, protein and phosphorus and other nutrients to the feeding animal. Pet foods, and animal feeds and corn food products made of the present invention will provide increased nutrition because of the increased bioavailability of the components of the grain.</p>			

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## **Animal Feed with Low Phytic Acid, Oil Burdened and Protein Laden Grain**

The present invention is based on and claims benefit to U.S. Provisional Application No.s 60/051,854 and 60/051,855, filed July 7, 1997, the entire contents of which are incorporated herein by reference.

### **Field of Invention**

Broadly, the present invention relates to grain having increased oil and increased protein and amino acids, increased total phosphorus bioavailability and decreased phytic acid. This grain can be used as feedstuffs for animals. More particularly, this invention relates to grain based feed that provide improved animal nutrition, and reduces the environmental impact of animal production.

### **Background**

Over the last fifty years, approaches toward providing animal nutrition have changed. No longer are the animals fed whatever grain or forage may be available. Instead, the diets of animals are closely monitored for total nutrition value, and for cost. The animal on the diet is monitored, for quality and performance characteristics, and for the environmental impact of the waste from the animal. The information gathered is employed to adjust the feed to increase nutrition value of the feed and the animal performance characteristics while decreasing the cost and environmental impact.

Cereals and fats are used in feeding programs for nonruminants such as swine and poultry to provide a nutritional source of calories.

The ratio of cereals to supplements, such as vitamins, minerals and fats, have changed across years in an attempt to maximize feeding efficiency of the animals. The feeding efficiency (the feed conversion ratio) or how much feed is required to produce one pound of animal weight is determined by a combination of matching the genetic potential of the animal, and the nutrients supplied to the animal. As the feed conversion ratio has risen due to genetic enhancements, the mineral nutrient requirements in the feed have risen to assure a complete and healthy diet.

Since an animal's ability to feed limits the amount of nutrients and calories it can consume, the feed industry has had to develop ways to make feeds that are more highly caloric. To increase the caloric density of the feed, producers have added fat to the feed. Fat has often been added to the feed in the form of a liquid. Fat has the advantage of supplying calories to each mouthful of feed. However, adding fat to feed has some disadvantages such as costs, added labor and technical difficulties with automatic feeding systems. Additionally, the fat is often of poor quality, thus reducing the overall quality of the feed.

To reduce the use of liquid fat in feeds, the industry has tried increasing the oil content of the grain used in the feeds. The Dupont company has developed and commercialized high oil corn as a method for increasing the oil content of feed. Other companies have developed corn that has more oil than no.2 yellow dent corn but less than Dupont's high oil corn. High oil and elevated oil corn is herein alternatively referred to as oil burdened corn. This extra oil in the corn reduces and may eliminate the need for the addition of the liquid fat to the feed.

Traditionally, oil burdened corn has been thought to contain increased level of phytic acid, as compared with levels in No. 2 yellow dent corn. Raboy et al (Journal of Heredity 1989: 80: 311-315) have reported however, that there is an apparent negative relationship  
5 between selection for oil and total phytic acid, phytic acid phosphorus and phosphorus per kernel, per germ and per endosperm of Illinois High Oil and Low Oil lines, as opposed to the previously expected apparent positive relationship on a concentration basis (i.e., mg constituent per g kernel, germ or endosperm). Raboy explains that the  
10 discrepancy between total contents per organ and concentration per organ results from the large divergence in organ dry weights exhibited between the Illinois High Oil (IHO) and Illinois Low Oil (ILO) seed used in his study; IHO germ being about twice the dry weight of ILO germ and ILO endosperm having nearly three times the dry weight of IHO  
15 endosperm. In contrast to this trend for high oil being linked to lower phytic acid, Raboy also reports a consistent positive relationship between increasing protein selection and increasing amounts of phytic acid, phytic acid phosphorus and phosphorus. Thus, there is an apparent positive relationship between selection for protein and total  
20 phytic acid, phytic acid phosphorus and phosphorus per kernel, per germ and per endosperm of Illinois high protein and low protein lines. This was maintained even when the data are expressed on a concentration basis (i.e., mg constituent per g kernel, germ or endosperm). Thus selection for protein and oil appears to divergently  
25 affect phytate content in seed.

As reports suggest an average increase of 0.38% protein with each 1% increase in oil (Han Y. Et al., 1987 Poultry Science 66:103-111; Keshararz, Poultry Pointers, pp6-7), it is uncertain from the art whether grains containing high oil, high protein and low phytic acid

could be produced (Brewer, "Optimum High Oil Corn Improves Poultry Rations" Poultry Digest, February/March 1998 pp30-31). Brewer states that while high oil corn is available as of 1998, varieties which are high in oil, high in protein and high in digestible phosphorus (i.e.,  
5 low in phytic acid phosphorus), have yet to be developed.

The concentration of phytic acid in grain-based diets has long been of concern to humans and animal nutritionists, because evidence has shown that phytic acid acts to form insoluble salts with nutritionally important minerals that subsequently are not absorbed in the intestine.  
10 Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)) is a form of phosphorus (P) in seeds which is stored in the form of phytate salts. Phytate salts have a negative nutritional impact on the animal because phosphorus bound to phytate is not available to the animal as a source of nutrition. Moreover, the animal does not retain  
15 the minerals such as Ca, Zn and the like and these needed minerals are excreted. Finally, the animal waste contain phytate P which then contributes to the surface and ground water pollution. If the grain is used for milling purposes then the milling by-products contain phytate P which then contributes to the surface and ground water pollution.

20 Swine, for example, lack the digestive enzyme (phytase) required to cleave the phosphorus from the phytate molecule and thus can not readily use phytate-phosphorus. Increasing the availability of phosphorus by elimination of the phytate salts binding the phosphorus would enable a reduction in dietary total phosphorus content without  
25 jeopardizing the animal's health or production performance. Increasing the bioavailability of phosphorus results in a lower phosphorus content in the swine wastes, which is environmentally desirable.

In one attempt to release a portion of the phytate P present in maize and soybean meal the feed industry has added microbial

phytase to the feed of animals. This method of dealing with phytate in the grain appears to partially decrease the phosphorus excreted by the animal. This research apparently led to further methods of degrading phytate in feed. One method includes adding an enzymatic cocktail  
5 and *Aspergillus niger* mycelium to feed. These components function to hydrolyze phytate present in the corn-soybean diet. Turkeys fed the enzymatic cocktail and the fungal mycelium showed enhanced performance and retention of P and Ca. These feed studies were planned to dephosphorylate the corn and soybean based feeds prior to  
10 consumption by the animal and thus reduce the P excreted. This method of dealing with phytate in the grain has the distinct disadvantage of adding labor and cost to the feed.

Mogen, in US Patent No. 5,593,963, describes production of a temperature stable phytase enzyme from *Aspergillus* in a corn or soy  
15 seed through genetic engineering techniques. The genetically produced phytase was designed to reduce the phytic acid content in animal feed by degrading the phytic acid being released from the grain and thus decrease the level of phosphorus excreted by the animal.

Low phytic acid mutant yellow dent corn seeds have been  
20 produced by Raboy and described in U.S. Patent No. 5,689,054. This patent describes the discovery of a single gene, nonlethal lpa1 mutants in maize that cause the reduction of kernel phytic acid phosphorus by up to 95% over the wildtype phytic acid phosphorus levels. Raboy notes that while the mutants of his invention are phenotypically very  
25 similar to the wild-type, the mutants would need to be introduced in to a breeding program in order to introduce the low phytic acid trait in to a commercial line. Moreover, Raboy explains that the low phytic acid maize mutants of his invention are characterized by a small kernel dry weight reduction which could result in a reduction in productivity and



that homozygous mutants may reduce or eliminate agronomically important characteristics. As Raboy et al (Journal of Heredity 1989) has indicated that divergent selection for high protein consistently produces higher phytic acid lines, it is unclear how the lpa1-R and lpa2-R mutations described in the Raboy patent in yellow dent corn will interact with genes for high-protein and oil-burdened corn seed. Thus one could not have predicted with certainty whether it would have been possible to maintain a high-protein oil burdened seed in combination with a low phytic acid mutant.

Although the feed industry has addressed both the need for more energy in the feed and the need for less phytate-phosphorus, the feed industry has not addressed the need for a method of providing, in a cost efficient manner, both the high nutrient density (i.e., high protein and high oil) and the low phytic acid in feed. There is a need to reduce the amount of phytate salts formed in feed and increase the amount of energy in feed without having to add phytase and liquid oil to feed. There remains a need, which has not been addressed, for a grain having a combination of increased protein and oil burden and low phytic acid levels. To reduce feed costs in animal production requires a nutritionally dense material that is cost-effective and environmentally friendly. Additionally, there remains a need for a feed containing an oil burdened, protein laden corn with low phytic acid levels which can be used for milling or for feed purposes.

## Summary of the Invention

An object of the present invention is to provide a nutrient-dense grain that contains both high levels of energy, through oil and improved amino-acid content, through protein, and low levels of phytic acid.

Another object of the present invention is to decrease the phosphate and/or phosphorus excretion of animals consuming the feed while increasing the energy levels per daily feed intake and bioavailability of minerals and other nutrients.

5       An object of the present invention is to provide an animal feed that contains both high levels of energy through oil and protein and low levels of phytic acid.

      Still a further object of the present invention is to provide a highly nutrient dense feed source to livestock which has less phytic  
10       acid present than the same feed source when made with regular commodity corn (i.e., no. 2 yellow dent corns).

      Still a further object of the present invention is to provide a high energy feed source to livestock containing sufficient supplies of any rate limiting amino acid which has less phytic acid present than the  
15       same feed source when made with regular commodity corn (no. 2 yellow dent corns).

      It is another object of the invention to provide a method of reducing animal phosphorus waste and/or pollution, and subsequent algal and microbial blooms caused therefrom, which method includes  
20       feeding animals, such as pigs and chickens, the animal feed of the present invention.

      In one embodiment, the present invention provides a non-lethal, mutant seed or grain of a cereal plant species, such as corn (maize), rice, barley and soy, having at least about 5% by dry weight,  
25       preferably at least about 6%, alternatively at least about 7%, oil; at least 11% by dry weight, preferably at least about 12%, alternatively at least about 13%, protein; and at least about a one third (33%) reduction in dry weight in the phytic acid amount (as measured by any of total phosphorus, phytic acid or phytic acid phosphorus), preferably

at least about a one half (50%) reduction, alternatively at least about 60-70% reduction, relative to wild-type seed of said species. Where the seed of the present invention is corn, the comparison in reduction is preferably made relative to standard number (no.) 2 yellow dent  
5 corn.

In another embodiment, the present invention provides an increase in phosphorus availability of from 28% for yellow dent corn to greater than about 70%, preferably less than about 90%, alternatively about 80% to about 84-85%. Availability being the amount of utilizable  
10 phosphorus compared to total phosphorous from feed. The hybrid grain of the present invention is preferably a cross between useful inbreds and an inbred line ExSeed line U095 -lpa1-E (alternatively referred to as U095-E or U095py; deposited as strain designation EX1965py on July 7, 1998 with American Type Culture Collection,  
15 10801 University Blvd., Manassas, VA 20110-2209 USA, under conditions of the Budapest Treaty, Accession No. \_\_\_\_\_. Source U095-py 1656-W97 - Florida - 100) The "E" or "py" designation used herein indicates the introduction of a lpa1 mutation by the present inventors. A number of other crosses and inbreds can be employed.  
20 For example, the following female inbreds BD68py, TR306py, WD22py and TR329py were crossed with male inbreds U095py, UU01py, UE95py, TR335py and TR386py to make high-yielding hybrid combinations. Crosses with U095py are particularly preferred and the inbred U095py and hybrids made therefrom are specific embodiments  
25 of the present invention. The hybrid grain of the present invention characterized by having ~6% oil and 12% protein (or 3% more oil and 3% more protein than yellow dent corn) and at least about 33% reduction in phytic acid content.

In another embodiment, the present invention provides a feed containing a seed, as described herein, and at least one source of vitamins or minerals, containing, for example, any one or a mixture of at least two of calcium or phosphorus or salts thereof, vitamin A, vitamin D, vitamin E, B<sub>12</sub>, riboflavin, pantothenic acid, niacin, biotin, and trace minerals, such as iron, copper, manganese, zinc, iodine, and selenium, and/or additional feed additives, such as antibiotics, arsenicals, chemotherapeutics, flavoring, antioxidants and plant extracts; said feed providing a nutritionally balanced diet and a greater amount of biologically useful phosphorus to an animal consuming said feed than does the same feed formed with wild-type seed of the species. The feed of the present invention may also contain amino acid additives, such as lysine and methionine.

In another embodiment, the present invention provides an improved feed which is otherwise formulated for swine or poultry but includes the seed, preferably corn seed, of the present invention.

In yet another embodiment, the present invention provides a method of increasing bioavailability of phosphorus from products containing wild-type seed of a species, said method including the steps of providing a seed containing product, such as a feed as described herein, for consumption, wherein the seed containing product contains a seed of the present invention, and feeding the seed containing product to an animal which will benefit from an increased bioavailability of phosphorus.

In a further embodiment, the present invention provides germplasm which will yield the seed of the present invention. In a preferred embodiment, the present invention provides corn germplasm which will yield the corn seed described herein.

In yet another embodiment, the present invention provides a plant produced from a seed of the present invention.

In yet a further embodiment, the present invention provides a seed of the present invention which is fully mature.

5        Still, further objects and advantages will become apparent from a consideration of the ensuing description.

### **Brief Description of the Figures**

10        Figure 1 shows a frequency distribution of phytate contents of screened M2 seed.

### **Detailed Description**

15        The present invention provide grain, feed made from the grain, petfood made from the grain, and food products made from the grain. The grain is preferably maize grain with the following characteristics: oil burdened, elevated protein content, and low phytate levels. The preferred grain has at least 5% oil, at least 11% protein, and at least  
20        about 20% to about 70%, preferably at least about 33% to about 60% reduction in the phytic acid level relative to wild-type grain, such as standard yellow dent corn. More preferably the grain has at least 6%, more preferably 7% oil, at least 12%, and more preferably 13% protein, and at least a one half reduction in the phytic acid level relative  
25        to standard yellow dent corn wherein the grain is low phytate. Percentages are expressed on a dry weight basis as amount of a constituent per kernel, unless described otherwise. The combination of oil burdened, protein laden, decreased phytate characteristics in grain makes a grain that provides more calories, protein and

phosphorus and other nutrients to the feeding animal. Pet foods, animal feeds and corn food products made of the present invention will provide increased nutrition because of the increased bioavailability of the components of the grain.

5           In other words the present invention includes an animal feed for a specific animal type. In one embodiment, the present invention provides a feed having a gross energy and at least the same ratio of performance limiting amino acids to gross energy as a nutritionally balanced feed using no. 2 yellow dent corn formulated for a similar  
10   type animal. The feed is formulated with an energy source including elevated oil, protein and low phytic acid maize. Additionally, the feed can contain at least one protein source including a potentially performance limiting amino acid component in a ratio to said gross energy such that the amino acid is not performance limiting, at least  
15   one source of vitamins and minerals; wherein the feed provides to the animal a higher calorie and lower phytic acid grain than no. 2 yellow dent corn in a nutritionally balanced feed. The limiting amino acid can be various different amino acids according to the needs of the animal species but it preferably includes lysine, tryptophan, threonine and  
20   methionine. The protein source of the feed can also include soybeans as a component. The feed of the present invention can also include the vitamins and a mineral source such as calcium, phosphorus and salt. In one embodiment, the feed of the present invention has vitamins and mineral sources which include any one or a mixture of  
25   vitamin A, E, D, B<sub>12</sub>, riboflavin, pantothenic acid, niacin, biotin; trace minerals, such as any one or a mixture of iron, copper, manganese, zinc, iodine, selenium, and feed additives, such as are known in the art and may include any one or a mixture of antibiotics, arsenicals, chemotherap utics, flavoring, antioxidants and plant xtracts.

The present invention provides a method of increasing bioavailability of phosphorus from maize containing products comprising the steps of: providing a maize containing product for consumption, wherein said maize containing product is formed from  
5 maize grain of the present invention, such as that characterized by having at least 5% oil, at least 11% protein, and at least a one third reduction in the phytic acid level relative to standard yellow dent corn, wherein the grain of the present invention is lower in phytate concentration than standard yellow dent corn; and consuming said  
10 maize containing product which contains less phytate in the maize material than the same maize product made with yellow dent corn wherein the bioavailability of the phosphorus in said maize is increased over the same product made with yellow dent corn.

The present invention further provides a feed for any non  
15 ruminant animals. The feed of the present invention is particularly well-suited as a constituent in the diets of swine or poultry.

In another embodiment, the present invention provides an animal feed having a gross energy content for a specific animal type, said animal requiring a certain level of an amino acid in the feed to  
20 achieve good performance from said feed, the feed containing corn having an elevated amount of oil compared to the average oil levels of no.2 yellow dent corn and low phytic acid levels compared to the average phytic acid levels of no. 2 yellow dent corn; a protein source, preferably substantially provided from the grain of the present  
25 invention; and having at least the same ratio of performance limiting amino acid to gross energy as a nutritionally balanced feed using no. 2 yellow dent corn formulated for a similar type animal.

The present invention provides grain having increased energy, protein and low phytic acid. This grain can be used as feedstuffs for

animals or this grain can be milled. The present invention provides a new maize seed, plant and grain that carry the oil burdened, protein laden and the low phytic acid and the use for such new grain. The grain-based feedstuffs provide improved animal nutrition, and reduce the environmental impact of animal production. Even more particularly this invention provides an animal feed formulated using the grain of the present invention.

Many crop plants are used for the production of food for human or animal consumption, for commercial processes yielding products for human consumption, for the development of industrial products and for other purposes. Traditionally, the improvement of crop plant species involves the introduction of desired traits by genetic crosses. The present invention likewise can be made repeatedly through the use of standard crop, such as corn, breeding and mutation practices.

Corn grain is considered to be a high quality grain for use in foods and feeds. High oil corn is considered more energy dense than other corn. High oil corn presently is commercially available from Dupont. However, this corn like all corn contains phytic acid. Until the present invention low phytic acid and oil burdened, protein laden corn plants and grain did not exist. The invention also provides an improved flour from milling of the seed of the present invention. Thus, low phytate, oil burdened, elevated protein maize grain should address the need for energy, protein dense corn and mineral bioavailability within the cereal grain.

The method of repeatedly making the grain of the present invention is as follows. Generally, in the course of a maize breeding program oil burdened, protein laden corn plants are crossed with maize plants carrying the low phytic acid allele. Oil burdened corn plants can be developed by recurrent selection as evidenced by



sun(hiO)-sunflower high oil  
canol-canola  
IolInCan-low linoleic canola  
5 Crambe  
corn  
soy-soybean

Thus the present invention encompasses grain that is oil  
10 burdened, preferably with fatty acid compositions in the ratios listed  
above. The present invention also encompasses corn that has  
increased the fatty acid compositions such that there is not an overall  
increase but there is a result increase in usefulness of the material to  
the animal fed the altered corn. The present invention also  
15 encompasses grain that is oil burdened and the fatty acid compositions  
are in a different ratio than listed above.

The ratio of oils present in grain cereals can be varied though  
genetic mutation, selective breeding, transformation and the like. The  
essential grain for the feed can be produced by standard methods of  
20 production of hybrid material such as the crossing of a oil burdened,  
elevated protein inbred with a low phytic acid inbred to produce hybrids  
having the grain characteristics including the low fatty acid and the  
elevated oil.

As noted above, Raboy et al have reported a positive correlation  
25 between protein selection and increased levels of phytic acid in corn.  
The following table lists the value of the amino acids present in one  
type of nutrient dense corn which can be used to form the grain of the  
present invention. The protein in corn is controlled by many different  
genes. The preferred high protein corn has an increased percentage  
30 of most of the amino acids present in corn grain. One example of high

prot in material has been the high protein corn developed by recurrent selection in the University of Illinois. Many of the other high protein corn available have an increase in lysine and not necessarily an overall increase in most amino acids. This type of high protein is also useful as this increased protein is needed by the feeding animal for overall health and this protein is better utilized when combined with the other traits of the present invention. To facilitate the breeding of hybrid seed the high protein trait is additive or dominant. Quality protein maize includes the high protein material available from Wilson seeds and from Crows and from public depositories and universities. Alternatively, high protein material can be generated by recurrent selection or by the use of certain mutation in corn like o2:su2 which have 49% more lysine than normal hybrids but have decreased protein yields. In development of the present invention a high protein trait that was additive and increased most protein levels beyond average yellow dent corn was used. Protein laden corn shall refer to corn that has increased amino acid content when compared to average amino acid content of yellow dent corn, and/or differing ratios of amino acid contents when compared to average amino acid content of yellow dent corn. High protein corn shall refer to corn that has increased amino acid content when compared to average amino acid content of yellow dent corn.

Table 2: Yellow-dent vs. EX404

( EX404 has ~1% wt% more oil and ~2-3% wt% more protein than does standard yellow dent corn (average 3-3.5% by weight oil and 7-9% by weight protein) EX404 and ES404 as used herein are the same)  
Amino Acid Composition:

		Dent	ES404	% change
	TRYPTOPHAN	.06	.06	100%
	ASPARTIC ACID	.57	.83	146%
	THREONINE	.29	.47	162%
5	SERINE	.41	.63	154%
	GLUTAMIC ACID	1.52	1.89	124%
	PROLINE	.74	1.04	141%
	GLYCINE	.33	.41	124%
	ALANINE	.63	.87	138%
10	CYSTEINE	.18	.24	133%
	VALINE	.38	.47	124%
	METHIONINE	.17	.26	152%
	ISOLEUCINE	.26	.36	138%
	LEUCINE	1.01	1.48	146%
15	HISTIDINE	.24	.35	145%
	LYSINE	.24	.31	129%
	ARGININE	.29	.42	107%

This table clearly evidences that most of the the amino acid  
 20 values of the nutrient dense corn are increased over the yellow dent  
 material. This extra protein appears to be available for the animal's  
 use when the phytic acid is substantially reduced. The ratio of proteins  
 present in grain cereals can be varied though genetic mutation,  
 selective breeding, transformation and the like. The present invention  
 25 provides a grain containing increased levels of bioavailable protein in  
 conjunction with increased levels of oil and reduced levels of phytic  
 acid.

Prior to the release by the USDA of the Low Phytic Acid mutants  
 B73 lpa1-R, A632 lpa1-R and sel ction thereof, there was only the

conventional method of breeding for producing low phytic acid in corn seed. Low phytic acid in corn seed developed by standard breeding appeared to carry some undesirable agronomic traits. Due to the recessive nature of the phytic acid gene the preferred method requires  
5 that this gene be fixed in both inbreds. The mutant containing low phytic acid can be developed according to the following method. Selection for phytic levels must be carefully performed as too low a level of phytic acid may result in lethal seeds.

The low phytic acid plant of the present invention can be  
10 developed by following the listed steps which do not take undue experimentation and can be done by the ordinarily skilled person in the art of plant breeding. The best method for generating a low phytic acid maize plant employs maize pollen mutagenesis. The induced mutation in a haploid pollen grain would give rise to a heterozygous genotype in  
15 the seed. Since low phytic acid presently is known as a recessive gene, the resultant mutant seed must be planted and selfed and the resultant plants seeds assayed for the mutant phenotype. The assaying of the seed should be done when the seed is in the mature stage or harvesting stage.

20 Mutagenesis is effected by conventional means in the art such as irradiation, chemical treatment, and transposable element insertion. One standard procedure is taught by Neuffer et al. Maize for Biological Research, W.F. Sheridan (Ed.), Plant Molecular Biology Association (1982). This procedure uses ethyl methane sulfonate (EMS) applied to  
25 pollen. The pollen is used for pollination and the resultant seeds are planted and the seeds from the second generation can be tested for phytic acid content. The test for P has been known in the art since 1990 when the HVPE method was published in Maydica 35:383 (1990) by Raboy. The method relies on differential migration of phosphorus

compounds. After electrophoretically fractionating the compounds a chromatogram allows a semi quantitative assessment of the phytic acid relative to other compounds. An alternative method involves screening for higher levels of inorganic P in the grain. For example grain samples can be ground (to pass a 2 mm screen in a Wiley mill) followed by addition of either 50 mg of grain germ or 1 gram of endosperm in 15ml of 0.4 M HCL in 0.7 Na<sub>2</sub> SO<sub>4</sub>. Phytic acid precipitates as an iron salt. Phosphorus in the ferric phytate precipitates and total P are determined. Phytic acid P (mg) are converted to phytic acid by use of a conversion factor of 3.5. These results lead to the selection of the desired maize plants containing the desired alleles. Other methods of testing for P are known and can be used to select plants. The seed containing the desired phytic acid is then increased. This process was employed in the present invention and inbred line were selected that carried new alleles at the lpa1 locus. These included EX404 (low phytic) which was crossed to one of the inbreds of the present invention to form a hybrid that produced the grain of the present invention. Additionally, the developed inbreds of the present invention were from stiff stalk, Lancaster and another versatile heterotic patterns so that the inbreds when crossed together with the appropriate heterotic groups formed excellent hybrid material. It was also discovered that a number of the developed mutations of the present invention, though low in phytic acid were not the same mutant as the lpa1-R mutation as indicated by allelic testing.

The method used in the present invention can be used to form two inbreds which would be crossed to form a high yielding hybrid. Inbreds are commercially available from Universities and Foundation Seed Companies and can be made by plant breeders skilled in the art of breeding maize inbred lines. One or both of the inbreds fixed for the

low phytic acid can be crossed to an inbred which carries the elevated oil trait. Either inbred can carry the high protein trait if it was selected as an additive trait. Thus allowing the hybrid combination to carry both traits the recessive low phytic acid and the dominant or additive oil genes. A corn plant is repeatedly bred until the low phytic acid allele is present in two inbreds that cross well to one another. Likewise at least one of the inbreds in the hybrid combination must contain the oil burdened trait and/or the protein laden corn alleles.

Alternatively this grain can be produced by using the crossing method of breeding taught in the Dupont patent application WO92/08341 (US 615,839). This method requires that the male pollinating plant carries the oil burdened trait. In Dupont's method the oil pollinator which is an inbred is mixed in with hybrid seed that is male sterile. The male pollinator then forms oil burdened grain on a sturdy hybrid plant.

In the present invention the male pollinator would preferably have two traits, a fixed recessive gene for phytic acid and the oil burdened, protein laden trait. Additionally, the male sterile hybrid would have the low phytic acid trait also.

Methods of formulating an animal feed that contains additional energy, protein and low phytic acid in one grain source are herein described. The preferred method is to improve feeds or petfoods by substituting the grain of the present invention for the corn grain normally employed. Other ingredients in the feed can be milling coproducts, protein ingredients, soybean meal, trace minerals and vitamins, and other cereals and feed additives and flavorings. This feed requires that grain be produced that is characterized as having increased oil and increased protein/amino acid and low in phytic acid. Surprisingly, the combination of the low phytic acid with the oil

burdened and elevated protein appears to allow the animal to utilize the extra protein that is in the oil burdened corn and the newly available phosphorus more efficiently leading to increased feed efficiencies. The grain resulting from this hybrid combination can be  
5 used in an animal feed. This feed should contain the combination of oil burdened, elevated protein and low phytic acid and other feedstuffs employed in the diets of either swine or poultry.

Preferably if other grains are used, these grains are selected to have as low a phytic acid content as is possible in that type of grain.  
10 Though the percentage of the grain as a part of the overall diet would determine how stringently the selection against phytic acid would have to be.

The present invention is of particular use for feeding non ruminant animals. The feed ingredients for the standard swine diet are  
15 provided below. The feed mixture of the present invention substitutes the grain of the present invention for the no. 2 corn. The grain having characteristics of low phytic acid and higher oil and increased amino acids. This grain, when provided to the animal, results in a higher caloric density available and a greater bioavailability of a number of  
20 nutrients such as minerals. The use of this grain in feed reduces the need for added animal fat or vegetable oils.

Swine are primarily fed a fortified corn-soybean meal diet. Piglets, pre-weaning or early weaned, often have additives in the diets such as dried milk products, and other high protein and fat sources  
25 added to the diet. A complete swine diet provides all the nutritional needs of the pig in one diet. These diets may be prepared by mixing a balanced supplement with corn: soybeans meal and a vitamin-mineral premix with corn. Conventional feeds were often supplemented with sources of supplemental fat which are plant seed oils (extracted),

grease and tallow, and commercial dry fat, corn oil, soybean oil or full  
fatted cooked soybeans. Flowability of the diet in automated feeders  
was compromised at 6 percent fat. Overall 1 percent fat produced  
approximately 2 percent improvement in efficiency (additional weight  
5 gain per weight of feed consumed). Increased oil from the use of  
the present feed (employing maize grain that is elevated oil and low  
phytic acid) increases the caloric density of the diet bioavailability of  
metals and reduces undesired waste. The feed of the present  
invention employs the corn grain of the present invention as a  
10 substitute for the grain presently employed in diets for animals. The  
following two new feed combinations will employ such grain. Both of  
these feed uses are for animals which are preferable non ruminant  
animals. Such animals include swine, poultry, cats, dogs, horses,  
sheep and the like.

15 Dietary nutrient density, when increased, maximizes animal  
performance. This includes animals such as sheep, swine, poultry,  
dogs, cats and horses. This grain is primarily effective with most  
monogastric animals. To formulate a diet containing the grain of the  
present invention that contains higher levels of oil and amino acid than  
20 standard corn requires the step of substituting the grain of the present  
invention for the yellow dent corn (or even for the oil burdened corn) in  
the diet. The invention then however, requires the step of adjusting the  
protein to energy level of the diet according to the needs of the animal.  
For example if the diet is for swine then the ratio of lysine should be  
25 increased. If the diet is for poultry then the rate of methionine should  
be increased and likewise for each animal.

The amino acid composition of protein specifically lysine which  
acts as a growth limiting amino acid for swine is increased in light of  
increased energy levels to keep a good balance of energy verses



protein when using the feed of the present invention. The invention itself increases the lysine. In the example given in table 2 of one of the maize grains containing increased oil and amino acid content the increase in Lysine is 29% over the regular maize grain. If the increase in the rate limiting amino acid in the grain of the present invention is sufficient, the diet may not require additional increases in this amino acid. However, to the extent that the grain does not carry all of the necessary amino acids in sufficient amounts to put the diet in balance, they must be added to the diet.

- 10 The following table includes ingredients that are used in a standard feed for swine. In the present invention these ingredients can likewise be used, however, the standard corn and corn products made from standard corn should be substituted by using the grain of the present invention or products made from the grain of the present invention. Preferably added fats are not required in the feed.
- 15 Additionally use of low phytic legumes and other protein sources are encouraged. These examples, as others herein, are illustrative of the use of the present invention and are not intended to limit the scope of the disclosed invention.

Energy, Fat, Protein of Typical Ingredients Used in Swine Diets			
Ingredient	kcal metabolizable energy/lb	protein %	fat %
Animal fat	3585		100
Alfalfa	775	17	3.0
Barley	1380	11	1.8
Blood meal	1060	86.0	1.0
Corn, yellow	1555	8.0	3.25
Corn, gluten (ml)	1760	60.0	1.0
cottonseed meal	1160	41.0	2.0

distillers dried grain w/ solubles	1515	27.0	8
fish meal	1500	60.0	6.0
Lysine hydrochloride, limestone, dicalcium phosphate	1100	48.0	5.0
Milo	1490	9.0	2.0
Molasses	910	3.0	
Oats	1243	12	3.0
Skimmed Milk (dried)	1630	33	1.0
Soybean meal	1535	47.5	1.0
Soybeans (fat cooked)	1650	37	18.0
Soybean Oil	3300		100.0
sunflower	1000	28.0	2.0
Wheat	1500	13	3.0
Whey	1405	14	.5

- As is clearly evidenced in articles such as Adams, K.L. and Jensen A.H. "High Fat Maize in Diets for Pigs and Sows in Animal
- 5 Feed" in Science and Technology, 17 (1987) 201-212, hogs fed higher energy diets using just high oil corn (and not the present invention of oil burdened, elevated protein and low phytic acid) with an energy: lysine ratio adjusted to the animal type (ie farrowing, starter pigs, finishing pigs) showed better feed efficiency then the same type of pig
- 10 on a No.2 corn soybean diet. The present invention used on this type of pig will provide a diet that is not only calorie dense and thus increases feed efficiency but also is low in phytic acid which permits the feeding animals to absorb from the grain the trace minerals, amino

acids and macro minerals needed for increased efficiency and gain. However, like the previous tests with high oil corn the pig on a diet including grain of the present invention will require the increase in nutrients from the oil burdened, elevated protein and low phytic acid  
5 grain to be balanced with an increase in the lysine level, if necessary.

Pig diets to be nutritionally balanced should have protein, amino acids, such as lysine, threonine, tryptophan, and major minerals, such as calcium, phosphorus, salt, and vitamins such as A, D, E, B<sub>12</sub>, Riboflavin, Pantothenic acid, Niacin, Biotin and trace minerals such as  
10 iron, copper, manganese zinc, iodine, selenium. Additionally feed diets often include feed additives such as flavorings and may have some of the following chemicals added Apramycin, bacitracin, chlortetracycline, bambemycin, carbadox, lincomycin, penicillin, tylosin, virginiamycin and the like.

15 Another potential use (other than as petfood and feed for swine) of the feed described herein is for poultry feed. Poultry includes most domesticated fowl including duck, geese, pheasant, turkeys and chickens. The nutrient requirements of poultry and swine differ but the basic ingredients in their diets are very similar. Poultry utilizes cereal  
20 grains and grain co-products, fats and carbohydrates in their diets. The largest part of the poultry diet is made up of cereal grain. These at least include corn, wheat, oats, milo, barley. However, in accordance with the present invention the preferred main grain ingredient is oil burdened, protein laden and low phytic acid corn. The present  
25 invention envisions that little to no corn is used in the feed of the present invention that is not the grain or a product of the grain of the present invention. In addition the feed of the present invention includes proteins which may come from peanut meal, soybean meal, cottonseed meal, fish byproducts, blood meal and poultry byproducts

and the like. The feed of the present invention may also include minerals and vitamins particularly calcium as limestone or oyster shells and vitamins D<sub>3</sub>, choline, menadione, A, E, B<sub>12</sub> and feed additives such as antioxidants, antibiotics. Like swine, poultry seem to have a specific amino acid that, if deficient, will reduce the animal's performance on the feed. For poultry, the limiting amino acid is methionine while for swine the limiting amino acid is lysine. Thus, when the present invention feed is formulated there must be an increase in the amount of methionine and general protein to keep the desired protein gross energy ratio in the diet as compared to the same ratio in the diet formulated with no. 2 grain maize. Again the present invention provides not only the nutrient dense feed but increases the bioavailability of the trace metals due to the decrease in the chelating agent, phytic acid, resulting in an increase in feed efficiency of poultry on the present invention.

Although the description above contains many specificities, these should not be construed as limiting the scope of the invention but as merely providing illustrations of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible within its scope.

The Iowa State University extension office publication entitled "Life cycle Swine Nutrition," PM489, revised 1996 on pages 12, 15 and 20 show a number of diets for sows and boars under standard production conditions. The following information shows that pig feeds and poultry feeds for different animals life stages are known in the prior art. The following is an example of one diet for a pig at a given development stage.

**Example 1**

A pig having a moderate, lean growth potential with the following weights are defined by the following stage development numbers. Pounds 6-8=stage 1, Pounds 8-13 =stage 2, Pounds 13-18= stage 3, Pounds 18-26= stage 4, Pounds 26-37= stage 5, Pounds 37-51== stage 6, Pounds 51-69=stage 7, Pounds 69-91= stage 8, Pounds 91-118= stage 9, Pounds 118-150 = stage 10, Pounds 150-188 = stage 11, Pounds 188- 233 = stage 12, Pounds 233-283 = stage 13.

The pig diet developed herein was formulated for a pig in stage six. A stage six pig would require for every one thousand pounds of feed: 648.4 lbs of corn, 320.0 lbs soybean meal, dehulled 8.50 lbs of limestone, 15.25 lbs. dicalcium phosphate, 4.10 lbs salt, 1.65 lbs trace mineral premix, 0.75 lbs Fat soluble vitamin premix, 0.65 lbs vitamin B premix, 0.50 lbs Biotin and Folic acid premix, 0.20 lbs choline premix and any feed additives approved by FDA. This feed is made according to the present invention by substituting the corn of the present invention into the feed instead of the no. 2 corn and increasing the level of lysine to match the increased energy in the diet to avoid the lack of lysine from limiting in the animal's growth. If the feed is pelleted then the vitamins should be increased approximately 20%.

Corn is the basic cereal grain used in animal feeds. Corn contains many of the essential nutrients that are required by the animal. The modern animal, with its tremendous genetic ability to deposit lean tissue versus fat, has a tremendous requirement for nutrients. As this demand for fat, has a tremendous requirement for nutrients, as this demand for increased nutrition, we have at the same time see a decline in feed intake, While animal genetics had and continue to improve carcass qualities by reducing total body fat, the

environmentalists have indicated that our concentrated animal feeding operations are polluting the ground and subsequent groundwater. Knowing that corn is the leading feeding ingredient used in animal production the present invention is a corn that contains concentrated  
5 nutrients, and nutrients that had higher biological availability to the animal. Such nutrients include protein, energy, amino acids and minerals. The key mineral, phosphorus, is essential for growth, health and production. Phosphorus in corn is only ~30% available to the animal because of the phytic acid content of the corn. However, the  
10 nutrient dense corn of the present invention has a greater availability of the phosphorus.

#### Example 2

This experiment determines the response of commercial broiler  
15 chickens to low phytic acid mutant yellow dent corn as compared to yellow dent corn.

The trials use commercial broiler chickens in a 13-day feeding study. The basal diet is an NRC (National Recommendation Committee) based corn diet. The treatment is a replacement of  
20 conventional yellow dent corn with low phytic acid hybrid grain obtained from a cross between B73 lpa1-R and an Exseed inbred line UU01-lpa1-E (alternatively referred to herein as UU01-E or UU01-py) which is a low phytic mutant of yellow dent corn.

All experiments were managed under the Good Laboratory  
25 Practices with animals welfare at the forefront. Feed and water is supplied *ad libitum*, body weight and feed intake is measured throughout the experiments, phosphorus availability is measured by means known in the art. Carcass evaluations, total body weight gain, feed conversion ratio, mortality, morbidity and intakes are reported.

The expected results are improved phosphorus availability of low phytic acid yellow dent corn versus yellow dent corn.

It is believed that the grain of the present invention in the diet will result in less excreted phosphorus. This will result in less pollution.

5 In this study, 8 day old New Hampshire x Columbian male chicks with an average initial weight of 73.7g were used to compare the phosphorus availability of low phytate yellow dent corn of the present invention compared to conventional corn (yellow dent). This experiment was a CRD; 7Tx4Rx5C, 13 day trial (7 treatments, 4  
10 replications, 5 chicks per treatment).

The basal diet was as follows: cornstarch/dextrose (2:1 ratio) to 100%; soybean meal 47.4%; soybean oil 5%; Limestone 1.2 %; Salt 0.35%; vitamin mix (A, D<sub>3</sub>, E, K, scotin, riboflavin, pantothenic acid, niacin, choline chloride, folic acid, thiamine, B<sub>6</sub>, B<sub>12</sub>) 0.35%; mineral mix  
15 (copper, iodine, iron, manganese, selenium, zinc) 0.15%; choline 60, 0.1%; DL-Met 0.25; flavomycin 0.05%. The basal diet contained 0.1% available phosphorus, 0.63% Ca and 23% CP. All percentages are on a weight basis.

The treatment design for this trial was as follows 1: Basal diet; 2:  
20 Basal plus 0.06% phosphorus (inorganic phosphorus) from potassium phosphate ( $\text{KH}_2\text{PO}_4$ ); 3: Basal plus 0.12% phosphorus from potassium phosphate ( $\text{KH}_2\text{PO}_4$ ); 4: Basal plus 20% conventional corn; 5: Basal plus 40% conventional corn; 6: Basal plus 20% low phytate corn; 7: Basal plus 40% low phytate corn.

Weight gain - 8 - 20 d (g)							
	1	2	3	4	5	6	7
R1	227.8	269.8	291.2	209.2	249.4	253.2	283.0
R2	216.8*	267.6	295.4	224.0	258.8	249.4	273.0*
R3	237.0	285.6	294.4	229.8	259.6	244.2	255.0
R4	224.6	258.4	300.2	232.8	236.8	241.6	269.6
mean	226.5 <sup>d</sup>	270.3 <sup>b</sup>	295.3 <sup>a</sup>	223.9 <sup>d</sup>	251.1 <sup>c</sup>	247.1 <sup>c</sup>	270.1 <sup>b</sup>
g/c/d	17.4	20.7	22.7	17.2	19.3	19.0	20.7
Pooled SEM = 4.6; LSD = 13.5; *chick removed or died; g/c/d = grams/chick/day							

Feed Intake - 8 - 20 d (g)							
	1	2	3	4	5	6	7
R1	346.4	383.2	417.8	317.0	354.2	358.8	367.8
R2	325.9*	397.6	428.4	332.2	368.6	350.6	365.6*
R3	353.0	415.4	414.6	337.6	371.0	338.4	355.6
R4	348.6	387.0	435.6	343.8	349.8	344.6	367.6
mean	343.4 <sup>d</sup>	395.8 <sup>b</sup>	424.1 <sup>a</sup>	332.6 <sup>c</sup>	360.9 <sup>cd</sup>	348.1 <sup>de</sup>	364.1 <sup>c</sup>
g/c/d	26.4	30.4	32.6	25.5	27.7	26.7	28.0
Pooled SEM = 5.3; LSD = 15.6; *chick removed or died							

Gain/Feed - 8 - 20 d (g)							
	1	2	3	4	5	6	7
R1	0.658	0.704	0.697	0.660	0.704	0.706	0.769
R2	0.665*	0.673	0.690	0.674	0.702	0.711	0.747*
R3	0.671	0.688	0.710	0.681	0.700	0.722	0.717
R4	0.644	0.668	0.689	0.677	0.677	0.701	0.733
mean	0.659 <sup>c</sup>	0.683 <sup>cd</sup>	0.696 <sup>bc</sup>	0.673 <sup>de</sup>	0.695 <sup>bc</sup>	0.710 <sup>b</sup>	0.741 <sup>a</sup>
Pooled SEM = 0.006; LSD = 0.020; *chick removed or died							



Tibia Bone Ash (%)							
	1	2	3	4	5	6	7
R1	30.7	35.8	37.1	29.4	34.2	31.5	36.5
R2	30.2*	33.9	38.5	29.0	31.7	28.9	34.9*
R3	28.1	33.3	36.9	28.4	32.5	31.1	34.1
R4	30.2	33.7	36.6	29.1	31.0	29.7	35.0
mean	29.8 <sup>d</sup>	34.1 <sup>b</sup>	37.2 <sup>b</sup>	28.9 <sup>d</sup>	32.3 <sup>c</sup>	30.3 <sup>e</sup>	35.1 <sup>b</sup>
Pooled SEM = 0.5; LSD = 1.5; *chick removed or died							

Tibia Bone Ash (g)							
	1	2	3	4	5	6	7
R1	0.242	0.318	0.427	0.215	0.262	0.283	0.338
R2	0.230*	0.312	0.418	0.232	0.296	0.250	0.345*
R3	0.239	0.327	0.394	0.233	0.270	0.253	0.327
R4	0.241	0.300	0.390	0.248	0.254	0.261	0.339
mean	0.238 <sup>c</sup>	0.314 <sup>c</sup>	0.407 <sup>a</sup>	0.232 <sup>c</sup>	0.270 <sup>d</sup>	0.261 <sup>d</sup>	0.337 <sup>b</sup>
Pooled SEM = 0.006; LSD = 0.019; *chick removed or died							

- 5            Slope ratio and standard curve analysis from the above results indicates that the low phytate corn contains 3 times more available phosphorus than in conventional corn.

### Example 3

- 10           The method of producing these elite, agronomically sound and high yielding mutants is a known method called mutagenesis. The process is outlined in the Neuffer paper Maize Genetic Newsletter 45:146. It should be noted that EMS is a mutation process. Like all mutation processes the act of mutation can adversely effect the
- 15           agronomic traits especially yields of the plant. However, the starting

germplasm is superior to that in which the phytate mutant was previously formed. Thus the overall agronomic traits of the plant of the present invention are more easily preserved and selected for than the industries approach of recurrent selection or backcrossing. Mutations  
5 were induced in the inbred line by treating pollen with ethyl methane sulfonate in paraffin oil according to the procedure described by Neuffer (1974). This treatment was performed on a number of inbreds from the various plant genotypes of cereal. This example will focus on the development of maize low-phytate mutants by this process. This  
10 mutagenesis process has been used to make a number of cereal mutants.

The general steps of the process of the present invention include treating inbred pollen (in this case maize) with ethyl methane sulfonate hereinafter "EMS". Inbred pollen is placed in EMS in oil for  
15 45 minutes. A paint brush is used and the pollen is brushed on to the silks of a receptive corn ear. This forms the Mutant-1(M1) seed. Such seed are grown and self-pollinated to produce the Mutant-2 (M2) kernels. The resulting M2 kernels are tested for the low phytate phenotype.

20

#### Example 4

The HVPE method is a common test for low phytate mutants. (Raboy, Mydica 35:383 (1990)). The method relies on differential migration of phosphorus compounds. After electrophoretically  
25 fractionating the compounds a chromatogram allows a semi quantitative assessment of the phytic acid relative to other compounds. An alternative method is screening for higher levels of inorganic P in the grain. For example grain samples can be ground (to pass a 2 mm screen in A Wiley mill) add either 50 mg of grain

germ or 1 gram of endosperm in 15ml of 0.4 M HCL in 0.7 Na<sub>2</sub> SO<sub>4</sub>. Phytic acid precipitates as an iron salt. Phosphorus in the ferric phytate precipitates and total P are determined. Phytic acid P (mg) are converted to phytic acid by conversion factor 3.5.

5       The principles of phytate measurement are known. In the method employed in the present study, a solution of 5-sulfosalicylic acid and FeCl<sub>3</sub> (Wade reagent) forms a pink chromophore. Phytic acid binds iron in this solution decreasing the level of the pink color. The measurement of this loss of color can be used as an indication of  
10   phytic acid levels. Since the blank contains no phytate, all readings of samples that do contain phytate will be negative numbers. If there is too much phytate, however, the iron-phytate complex can precipitate as a milky white substance. In this case the pink color will not be present but the milky white matter will absorb light and result in falsely  
15   high readings. Thus some visual observation may be necessary. This may necessitate using a smaller aliquot (less than 25 microliters) of the corn extract if the corn variety has high levels of phytate.

A rapid screening procedure, such as described as follows, may be used to score for putative low phytate seeds. In this  
20   procedure, a single edge razor is used to cut the kernel tip cap off just behind the black layer. The cut should transect the scutellum at a point at or near the radicle tip. Ususally, 8 representative kernels were selected from each ear. The kernels are then placed, cut surface up, on a microplate that has the surface covered with  
25   cellophane tape (sticky side up). The staining procedure was completed after dissection of at least 100 families.

Staining was done with the use of a repeating pipette to place a 10 microliter drop of Wade reagent (as described below) on the cut surface of each kernel. After a few minutes the color disappeared as

the phytic acid from the scutellum binds the iron in the Wade reagent.

Observations were made for families that segregate for slower disappearance of the pink color relative to the others (perhaps 5 % of the total). These slower families were re-analyzed by the quantitative procedure described herein.

Phytate was quantified as follows. Individual kernels (7 to 10 from each family) were crushed in steel plate of a Carver hand-pump press (best results obtained when wells of plate lined with glycine paper and crushed with about 5000 lbs. pressure) and placed into 1.5 ml microcentrifuge tubes. 1 ml of 0.65 N hydrochloric acid was added and allowed to stand overnight. The combination was mixed by inversion the next day and allowed to settle for 5 minutes. A 15 microliter aliquot of the supernate was added to a microfuge tube with 100 microliters of buffer A (as described herein) and mixed. Low phytate mutants turn a very blue color due to the high phosphorus levels of the seeds. Mutants were generally retested the following day. The 0.65 N HCl extraction solution was made by adding 216 ml of 12.1N HCl to 3784 ml water. Reagent A was made fresh daily and included 2 parts (by volume) deionized water, 1 part (by volume) ascorbic acid solution, 1 part (by volume) ammonium molybdate solution and 1 part (by volume)  $\text{H}_2\text{SO}_4$  solution. Ammonium molybdate solution was made by adding 25 g  $(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \times 4\text{H}_2\text{O}$  to make 1L with water.  $\text{H}_2\text{SO}_4$  solution was made by adding 167 ml of 36 N sulfuric acid to 833 ml water. Ascorbic acid solution was made by adding 100 g L-ascorbic acid to make 1L with water. Ascorbic acid solution was stable with refrigeration for about seven weeks but only about two hours unrefrigerated.

Phytic Acid standards were prepared by means known in the art.

Maize kernel (M2) free phosphate was visually screened as follows. Kernels were selected, the phenotypes noted and placed in a multiple well crushing plate. Kernels were crushed in the multiple well crushing plate using a hydraulic press. Crushed kernels were  
5 transferred into 1.5 ml eppendorf microcentrifuge tubes. 0.5 ml of Reagent A was added. After allowing 2 hours reaction time, 0.5 ml reagent B was added. The tubes were capped and mixed by inverting. The reactions were scored visually for blueness after 1 hour, using a light box where necessary, and the bluest samples were selected as  
10 having highest phosphate. Often, the bluest samples from each family (ear) were selected and compared for final selection. Reagent A of this assay was prepared from 50 ml DMSO and 50 ml Reagent B. Reagent B was made fresh daily and prepared from 60 ml distilled water; 30 ml of 10% ascorbic solution (10 g ascorbic acid water to 100  
15 ml total volume; the ascorbic solution was refrigerated and stable for 1 week); 30 ml of 3.5% ammonium molybdate solution (2.5 g  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  add water to 100 ml total volume); 30 ml of 6N sulfuric acid solution (170 ml water plus 25 ml concentrated  $\text{H}_2\text{SO}_4$ , adjust to 200 ml total volume with water).

20 Phytate (Red Test) was quantitatively measured as follows. Approximately 12 mature seeds were crushed in a steel crush plate of a Carver hand-pump press. Best results were obtained when wells of the plate were lined with weighing paper. Kernels were crushed with 5000 to 10000 lbs. pressure and transferred to Eppendorf tubes. 1 ml  
25 of 0.65 N HCl was added to same, allowed to sit overnight and mixed the following day by tube inversion. To assay, 200  $\mu\text{l}$  of Wade-A reagent (described below) was combined with 10  $\mu\text{l}$  of the above obtained corn/HCl juice extract in individual wells of a microtiter plate. Any change in color was noted and samples which remained red

were noted as low in phytate since phytate binds with iron and turns the solution white. Quantitation may be completed with a spectrophotometer measuring at 490 nm. Wade-A reagent used herein was prepared by adding 25.4 g of 5-sulfosalicylic acid and 350 mg of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (ground with mortar and pestle if necessary) to 1.5 L deionized water. NaOH was used to adjust the pH to 3.05 and volume adjusted with d. $\text{H}_2\text{O}$  to 2L. This reagent was stable in a refrigerator for about 1 mo. 0.65N HCl was prepared by adding 216 ml HCl (12.1N) to 3784 ml of d. $\text{H}_2\text{O}$ .

10        These assays allow the selection of the desired maize plants containing the desired alleles. Other methods of testing for phosphorus are known and can be used to select plants.

Seed containing the desired levels of phytic acid are then increased. This process was employed in the present invention and a number of inbred lines were selected that carried the low phytic acid mutation. These included several low phytate inbred lines with good combining ability which were crossed together to form a hybrid that produced the grain of the present invention. Thus, the developed inbreds of the present invention were produced from stiff stalk, Lancaster and another versatile heterotic patterns so that the inbreds when crossed together with the appropriate heterotic pattern formed excellent hybrid material. It was also discovered that a number of the developed mutations of the present invention though low in phytic acid were not the same mutant as the *lpa1-R* mutation. Additionally, the seed were screened for germinability in standard seed germination tests. It was found that some low phytate mutants were unable to germinate whereas others would germinate normally. Only the seed with good germination characteristics were maintained.

Figure 1 shows an example of some data obtained from 3 such inbred lines screened for phytate content. Plotted as a frequency distribution curve it is clearly evident that there were a few samples with very low phytate content (less than 0.5 units (weight percent) whereas the bulk of the samples were higher in phytate content. The line known as UO95 line was selected as a starting material for its higher than average protein and oil levels. This line produces seed which are oil burdened and protein laden and which germinate normally. UO95py retains these characteristics with the low phytate levels described above. When crossed with certain other inbred lines, the resulting hybrid produces grain which are oil burdened and protein laden and contain low phytic levels.

The following table represents phytic acid contents (mg/g of seed) for mutant corn according to the present invention compared with wild-type seed.

No. Seeds	Wild-type Line	Phytic acid	Mutant Line	Phytic acid	% reduction
24	UU01	1.16	UU01-py	0.17	85.3
12	UO95	1.85	UO95-py	0.14	92.4
12	WD22	1.45	WD22-py	0.05	96.6

The following provides an example of an inbred line according to the present invention.

Inbred	Protein	Oil	Phytate	% phytate reduction
Wild-type				
UO95	13.4	606	1.85	
UU01	12.7	2.9	1.16	
B73	11.3	4.4	—	
WD22	—	—	1.45	

Mutants				
UO95py	14.4	5.3	0.14	85.3
UU01py	12.2	3.1	0.17	92.4
B73lpa1-R	13.2	3.2	—	—
WD22py	—	—	0.05	96.6

Protein and oil contents were measured by NIR analysis on a Dickey-John Reflectance Near Infra Red Spectrometer.

5           The grain of the present invention can also be used as a substitute source for the corn grain or flour used to make corn tortilla, corn meal, and cornflakes by substituting the grain of the present invention in the recipe and baking or processing as one would normally.

10           The grain of the present invention can also be used as a substitute for the corn wet milling industry by substituting the grain of the present invention in order to increase milling efficiency and recoverable starch content. Animal feed made as a by-product of the milling process is also substantially reduced in phytate content.

15

\* \* \* \* \*

The entire contents of references referred to herein are incorporated in their entirety by reference.

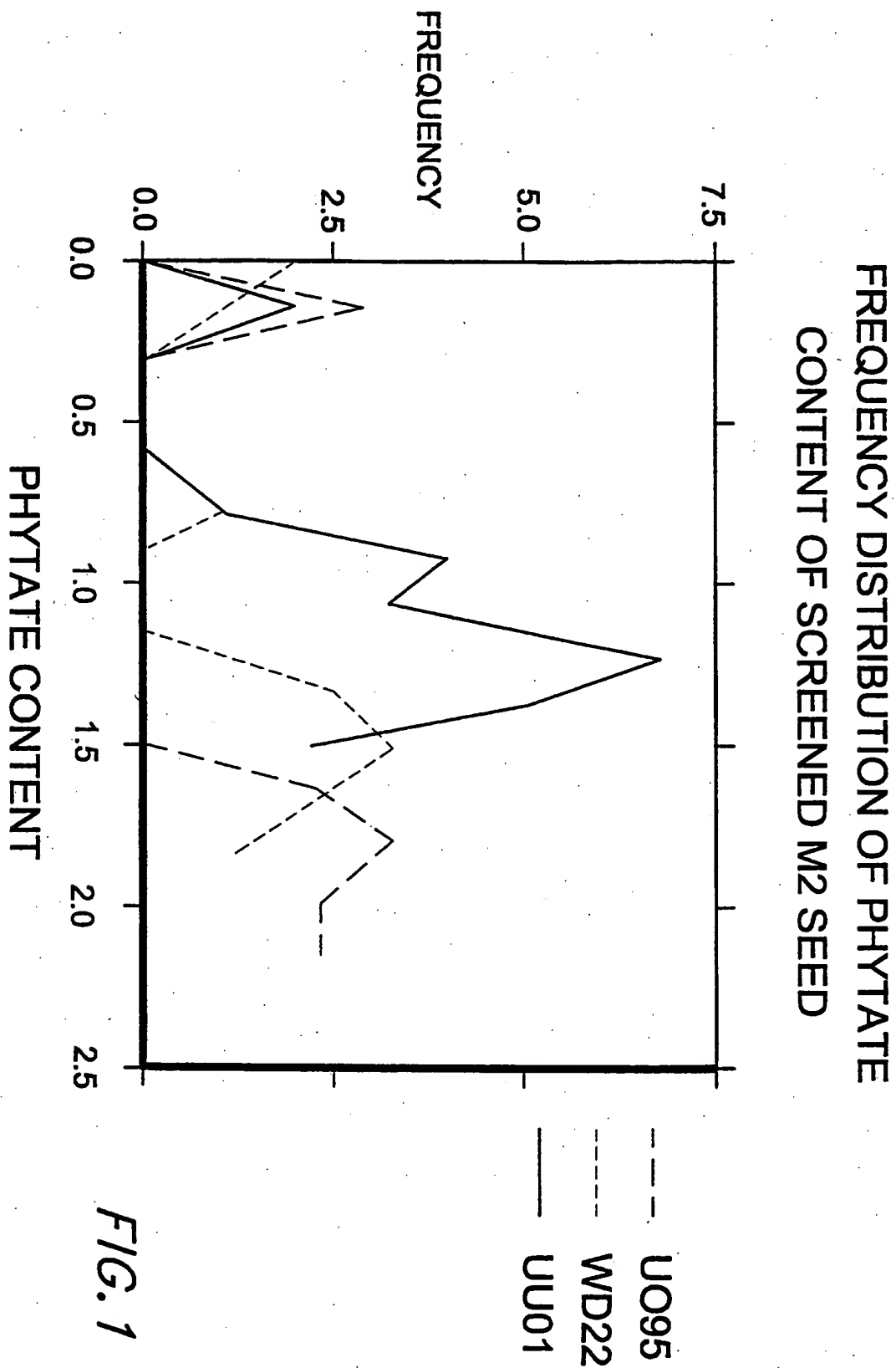


We claim:

1. A non-lethal, mutant seed of a cereal plant species having at least 5% by weight oil, at least 11% by weight protein, and at least a one third reduction in the phytic acid amount relative to wild-type seed of said species.
2. A seed according to claim 1 having at least 5% oil and at least 13% protein and said reduction in the amount of phytic acid is at least half relative to wild-type seed of said species.
3. A feed comprising a seed according to claim 1, and at least one source of vitamins or minerals; said feed providing a nutritionally balanced diet and a greater amount of phosphorus to an animal consuming said feed than does the same feed formed with wild-type seed of said species.
4. A feed comprising a seed according to claim 2, and at least one source of vitamins or minerals; said feed providing a nutritionally balanced diet and a greater amount of phosphorus to an animal consuming said feed than does the same feed formed with wild-type seed of said species.
5. A feed according to claim 3 wherein the vitamins and mineral source includes calcium, phosphorus and salt.
6. A feed according to claim 3 wherein the vitamins and mineral source includes at least one of vitamin A and vitamin D.

7. A feed according to claim 3 wherein the vitamins and mineral source includes vitamins selected from the group comprised of: vitamin E, B<sub>12</sub>, riboflavin, pantothenic acid, niacin, biotin.
8. A feed according to claim 3 wherein the vitamins and mineral source includes trace mineral selected from the group comprised of: iron, copper, manganese, zinc, iodine, selenium.
9. A feed according to claim 3, further comprising feed additives selected from the group comprising: antibiotics, arsenicals, chemotherapeutics, flavoring, antioxidants and plant extracts.
10. A feed according to claim 3 wherein the feed is supplemented with the amino acid lysine.
11. A feed according to claim 3 wherein the feed is supplemented with the amino acid is methionine.
12. A feed according to claim 3 wherein the components are formulated for the dietary requirements of swine.
13. A feed according to claim 3 wherein the components are formulated for the dietary requirements of poultry.
14. A seed according to claim 1 selected from the group consisting of maize seed, rice seed, soy seed and barley seed.

15. A method of increasing bioavailability of phosphorus from products containing wild-type seed of a species, said method comprising the steps of providing a seed containing product for consumption, wherein said seed containing product comprises a seed of claim 1, feeding said seed containing product to an animal which will benefit from an increased bioavailability of phosphorus.
16. Germplasm which will yield the corn grain of claim 1.
17. A plant produced from a seed according to claim 1.
18. A seed according to claim 1 wherein said seed is fully mature.
19. Inbred line UO95py
20. A hybrid formed from crossing inbred UO95py with an inbred corn line.
21. A seed according to claim 1 having at least 6% oil and at least 9% protein and said reduction in the amount of phytic acid is at least half relative to wild-type seed of said species.
22. A non-lethal, mutant seed of a cereal plant species having at least 5% by weight oil and at least a one third reduction in the phytic acid amount relative to wild-type seed of said species.
23. A seed according to claim 1 having at least 6% oil and at least 9% protein and said reduction in the amount of phytic acid is at least half relative to wild-type seed of said species.





Applicant's or agent's file reference number : 1-14	International application: PCT/US98/13685
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>7</u> , line <u>5</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution ATCC	
Address of depositary institution (including postal code and country) 10801 University Blvd Manassas, VA 20110-2209 USA	
Date of deposit July 7, 1998	Accession Number 203034
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

For receiving Office use only	
<input checked="" type="checkbox"/> This sheet was received with the international application	
Authorized officer 	

For International Bureau use only	
<input checked="" type="checkbox"/> This sheet was received by the International Bureau on:	
02 NOV 1998	
Authorized officer 	

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/13685

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 15/00, 15/01; A01H 1/00; A01H 1/06; A01H 5/00

US CL : 800/235, 250; 435/172; 47/58

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/235, 250; 435/172; 47/58

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, AGRICOLA

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RABOY et al. A Survey of Maize Kernel Mutants for Variation in Phytic Acid. Maydica. 1990, Vol. 35, pages 383-390, see the entire document.	1-23
Y	JONGBLOED et al. Apparent Digestible Phosphorus in the Feeding of Pigs in Relation to Availability, Requirement and Environment. 1. Digestible Phosphorus in Feedstuffs from Plant and Animal Origin. Netherlands Journal of Agriculture Science. 1990, Vol. 38, pages 567-575, see the entire document.	1-23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 OCTOBER 1998

Date of mailing of the international search report

22 OCT 1998

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/13685

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RABOY et al. Phytic Acid Levels in Seeds of Glycine Max and G. Soja as Influenced by Phosphorus Status. Crop Science. 1993, Vol. 33, pages 1300-1305, see the entire document.	1-23
Y	YI et al. Improving Phytate Phosphorus Availability in Corn and Soybean Meal for Broilers Using Microbial Phytase and Calculation of Phosphorus Equivalency Values for Phytase. Poultry Science. 1996, Vol. 75, pages 240-249, see the entire document.	1-23

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/13685**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/13685

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This International Search authority has found 3 inventions claimed in the International Application covered by the claims indicated below:

Group I. Claims 1, 2, 14, 16-23 are drawn to a non-lethal mutant seed of a cereal plant species.

Group II. Claims 3-13 are drawn to a feed.

Group III. Claim 15 is drawn to a method for increasing bioavailability of phosphorous from products.

The inventions listed as groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The specific technical features of group I is the characteristics of the mutant seed of a cereal plant species described in claim 1. The invention of group II does not require special technical features of group I because it is broader in scope than Group I and does not require the particular mutant required by Group I. The invention of Group III does not require the special technical features of Group I and II because the invention of Group III is drawn to a method of increasing bioavailability of phosphorous.

The claims are not so linked by a special technical feature within the meaning of the PCT Rule 13.2 so as to form a single inventive concept, accordingly, the unity of invention is lacking among all groups.